EXCEPTIONAL CASE REPORT

blood advances

Single-cell DNA sequencing reveals complex mechanisms of resistance to quizartinib

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Key Points

- Single-cell sequencing exposes previously unmeasurable complexity of tumor heterogeneity and clonal evolution on quizartinib.
- Single-cell sequencing reveals on- and off-target mechanisms of resistance to quizartinib, which can preexist therapy.

Introduction

Acute myeloid leukemia (AML) with activated Fms-like tyrosine kinase receptor III (*FLT3*) causes significant mortality secondary to relapsed/refractory (R/R) disease.[1-5] Drug resistance limits the duration of response of FLT3 inhibitors (FLT3i). Genetic evolution leading to R/R disease is incompletely understood, and leukemia's heterogeneity is incompletely described, even with next-generation sequencing (NGS). A deeper understanding of cellular heterogeneity is essential because intratumoral heterogeneity is ubiquitous, has prognostic relevance, affects response to therapy, and drives therapeutic resistance.[6-9] We characterized, via single-cell sequencing (SCS), the genetic evolution of R/R AML on the FLT3i quizartinib.

Case description

We analyzed serial samples from patients with R/R FLT3-positive internal tandem duplication (ITD^+) AML on phase 2 or 3 quizartinib monotherapy trials.[10,11] After treatment, 3 of 8 patients had complete response with incomplete hematologic recovery, 2 patients obtained a partial response, and 3 patients had no response; no patients obtained complete response.

Methods

We analyzed sixteen samples from bone marrow or peripheral blood from 8 patients (Table 1; supplemental Table 1). Included patients relapsed after initial response and had serial samples available for analysis.

We performed single-cell DNA sequencing on unsorted mononuclear cells using the Tapestri platform (Mission Bio, Inc).[12] This consisted of targeted sequencing of mutational hotspots using 40 amplicons from 19 AML-specific genes as previously described.[13] Data from 2 × 150 bp paired-end FASTQ files generated by Illumina HiSeq4000 was processed. For analysis, the Tapestri Insights software, a custom GATK-based variant-calling workflow,[14] the Integrative Genomics Viewer,[15] and the Maftools R scripts were used. Single-cell phylogenies and population hierarchies were reconstructed. Further details are provided in supplemental Methods.

Results and discussion

SCS provides a sensitive description of cellular populations compared with bulk NGS

We analyzed 103 031 cells from 16 timepoints from 8 patients. Patient characteristics are provided in Table 1; additional clinical information is provided in supplemental Table 1. Targeted SCS identified pathogenic variants not detected by clinical bulk NGS (supplemental Table 2). SCS revealed details of

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All genetic data for the dbGaP study can be accessed at the NCBI Sequence Read Archive (SRA) under accession no. phs002320.v1.p1 (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs002320.v1.p1). All code for custom sequencing analysis is uploaded to GitHub and/or published in open source publications. For original data, please contact the corresponding author at catherine.smith@ucsf.edu.

The full-text version of this article contains a data supplement.

Table 1. Patient characteristics

Clinical patient ID	ELN risk* at diagnosis	Cytogenetics at study entry	Best response	Duration of response, wk	Sample timepoint	No. of single cells analyzed	No. of clones
1	Adverse	46,XY,del(5)(q23q33)[5]/46,XY[4]	PR	5	Pre-quizartinib	13 538	6
					Relapse	4 734	6
2	Adverse	47,XY,+11[15]	PR	6	Pre-quizartinib	637	3
					Relapse	3 245	6
					Second relapse†	1 056	6
3	Adverse	46, XX	CRi	6	Pre-quizartinib	11 518	6
					Relapse	4 113	3
4	Adverse	46, XX	CRi	3	Pre-quizartinib	5 557	3
					Relapse	4 741	4
5	Favorable	Unavailable	CRi	10	Pre-quizartinib	9 774	4
					Relapse	6 867	8
6	Adverse	47, XX, +8, t(x;10)	None	NA	Pre-quizartinib	6 537	4
					Relapse	7 961	7
7	Unknown	47,XX,+8[1]/47,idem,del(16)(q13)[19]	None	NA	Pre-quizartinib	5 285	5
					Relapse	10 347	13
8	Unknown	47,XX,+8[3]	None	NA	Relapse	7 121	4

CRi, complete response with incomplete hematologic recovery; NA, not available; PR, partial response.
*Per European LeukemiaNet 2017 Guidelines.[21]
†Patient received quizartinib + chemotherapy between first and second relapse.

ITD number (3 patients had >1 ITD) and zygosity (supplemental Table 3). Samples were notably polyclonal: a mean of 6 distinct clones per sample (range, 3-13) with a mean of 5 clones before treatment and 7 clones at the time of relapse (Figure 1A; supplemental Table 2).

Quizartinib drives clonal selection for preexisting off-target mutations

SCS reveals selection of previously undetectable preexisting subclones containing off-target (non-FLT3) mutations. Two patients (Figure 1B-C) relapsed with off-target Ras pathway mutations, which have been previously associated with clinical gilteritinib and crenolanib resistance,[13,16] but not with quizartinib resistance. These data suggest that *RAS*-mutant cell populations expand on quizartinib. However, we acknowledge that secondary polyclonal *RAS* mutations which cooccur in the *FLT3* mutant clone[17] may behave differently than those in separate subpopulations. One of these patients (Figure 1B) had a predominant *NRAS*-mutant population at relapse in the setting of polyclonal off-target resistance clones: 2 *NRAS* mutations (at the G13 and Q61 loci) in separate cell populations as well as an additional *KIT* mutant population. All of these populations expanded on quizartinib monotherapy. *KIT* mutations have not been previously associated with quizartinib resistance. The second patient (Figure 1C) had expansion of both preexisting off-target, *KRAS* G13D, and on-target, *FLT3* N841, mutations, confirming our previous observation that on- and off-target mechanisms of resistance can coexist within the same leukemia.[18] At relapse, the dominant population contained a previously undetected *FLT3* D835Y, demonstrating that the preexisting subclone will not always drive resistance.

Quizartinib drives clonal selection for on-target FLT3 KD mutations

Seven of 8 patients developed at least 1 additional FLT3 kinase domain (KD) mutation, most commonly at the D835 locus (Figure 1C-H).[19,20] SCS reveals how KD mutations segregate with driver ITD and other mutations. FLT3 KD mutations can occur on the native (FLT3-ITD negative) allele (Figure 1C-D), in *cis* with the FLT3-ITD allele (Figure 1E-G), or in a combination of both (Figure 1H).[18] Quizartinib may select for preexisting KD mutant populations in native FLT3 alleles (Figure 1C) or KD mutant cells may appear de novo to dominate at the time of relapse (Figure 1D).

In addition to clonal selection, quizartinib drives clonal evolution of on-target FLT3-ITD KD mutations.

Within a FLT3-ITD population that dominates at relapse, SCS shows gain of FLT3 KD mutations in *cis* with ITD (Figure 1E-G; supplemental Figure 1). In addition to co-mutation with ITD, multiple KD mutations can coexist with each other within a single cell (Figure 1H), suggesting previously unmeasurable complexity, even at a single tumor-relevant locus.

Quizartinib drives complex evolution, which may lead to duplicative paths to resistance

Leukemias in 4 patients had polyclonal KD mutations. In all cases, the same KD mutations were present in multiple clones at same timepoint (Figure 1C,F-H). Though we cannot definitively rule out loss of heterozygosity, there is apparent parallel acquisition of these mutations in multiple patients (Figure 1F-H), emphasizing the importance of KD mutations in resistance as well as the leukemia's persistent dependence on FLT3 signaling. This is further highlighted by the fact that KD mutations may occur as heterozygous or

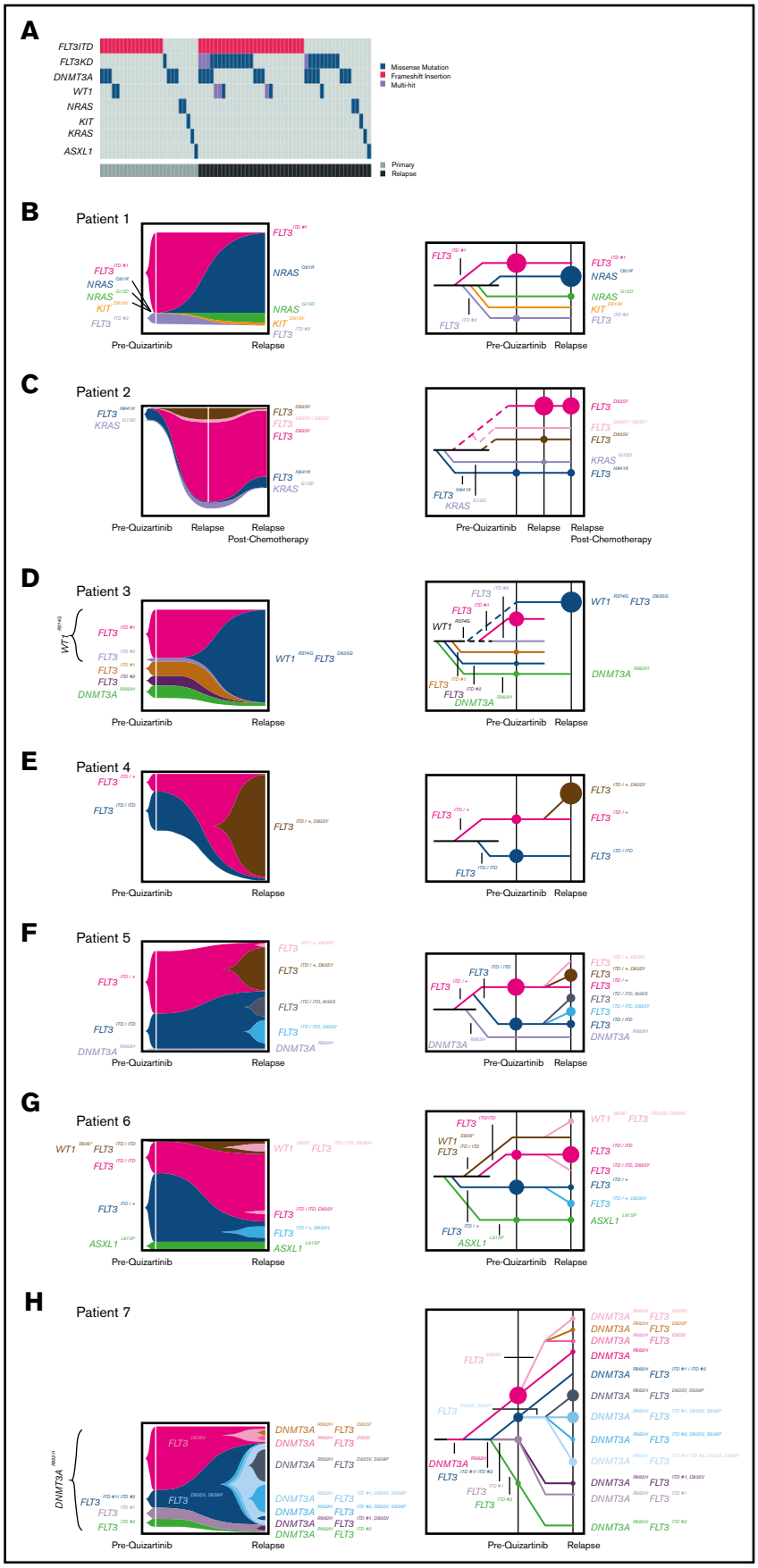

Figure 1. The genetic landscape of relapse on quizartinib monotherapy. Evolution represents 1 possibility based on detectable mutational data. (A) Overview of the mutational landscape on quizartinib monotherapy with somatic mutations shown across 70 unique clones (1 clone per column) from 16 patient samples. (B) Clonal selection of off-target mutations. Mutations in NRAS G13, Q61, and KIT D816 are detectable with SCS before initiation of therapy, each in <0.5% of the population, before expanding on quizartinib. RAS and KIT mutations have not previously been documented as clinical resistance mutations. (C) Complex on- and off-target resistance. This leukemia had a KD mutation at N841 and a KRAS mutation at G13, which were both detectable before therapy and expanded on quizartinib. At the time of relapse, this leukemia has gained 2 different KD mutations at the D835 locus, one of which is present in heterozygous and homozygous populations. (D) Clonal selection of FLT3 KD mutation. This patient relapsed with a single dominant clone with an on-target KD mutation at D835 in a non–ITD-containing population. This leukemia also has 2 different ITD mutations. (E) Clonal evolution with gain of FLT3 KD mutation. This patient relapsed with a single, dominant clone with a D835Y mutation in *cis* with FLT3-ITD. (F) Complex FLT3 evolution. Three different KD mutations emerge at relapse, including 2 at the D835 locus in different populations. The D835Y mutation arises in 2 separate populations. There are also mutations with hetero- and homozygous FLT3 ITD populations. (G) Complex FLT3 evolution. This patient relapsed with 2 different KD mutations, including a D835H mutation that arises in 2 different populations. There are also mutations with hetero- and homozygous FLT3 ITD populations. (H) Complex clonal evolution. At time of relapse, this patient had 13 clones, including 2 ITDs and 4 KD mutations; both SNV and MNV KD mutations may or may not be in FLT3-ITD^{+} clones.

homozygous alleles within the same leukemia (Figure 1C; supplemental Figure 2). The complexity of these KD mutations reveals more intricate tumor heterogeneity than previously appreciated, and also raises the question of whether certain leukemias are predisposed to mutate specific sites. One leukemia in particular highlights an extreme of mutational heterogeneity (Figure 1H) with 6 FLT3 aberrations in the same cell: 2 distinct ITDs (different insertion sites and lengths) and 4 different KD mutations including S838 and 3 multiallelic mutations at the D835 locus. Further, the D835V mutation occurred as both single- and multinucleotide variants (SNV and MNV) (ATC→AAC or CAC) (supplemental Figure 2). This patient exhibited additional MNVs creating the D835F and D835I alleles (supplemental Figure 2), which presumably arose as secondary mutations from the original D835V SNV (D835F: ATC→AAC→AAC; D835I: ATC→AAC→AAT). The striking duplicative polyclonality within this single locus implies that some AML patients may experience particularly high mutational burden including a predilection to mutations at specific resistance loci.

Serial SCS offers a novel look at the genetic evolution to relapse, with the ability to accurately determine zygosity, comutations, and clonal composition. Quizartinib binds FLT3 in its inactive conformation, leaving it vulnerable to resistance mutations that keep FLT3 in its active confirmation, like those in the TKD. This series corroborates FLT3 KD mutations as the most common mechanism of resistance to quizartinib; SCS further reveals that mutations may occur alone or in *cis* with ITD alleles. The ability to identify multiple KD mutations within the same leukemia, the same timepoint, the same cell, the same gene, and even the same locus, reveals striking biologic redundancy even within single cells. Additionally, we demonstrate for the first time that Ras pathway mutations are a mechanism of clinical resistance to quizartinib. Both off- and on-target mutations may exist before therapy. The ability to detect resistance mutations before overt clinical relapse and to visualize their expansion over time supports the need for effective combination therapy to suppress outgrowth of resistant clones. However, novel resistant subclones can arise to outcompete clones detectable before drug treatment; therefore, attempts to address only preexisting resistant clones may be insufficient to prevent relapse. In the future, SCS analysis may be used to prospectively monitor clonal populations on FLT3i treatment and facilitate dynamic adjustment of therapy.

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Authorship

Contribution: C.A.C.P., L.H.F.M., T.K., and H.J. performed processing of patient samples and sequencing as well as data analysis; B.J.H., R.D.-D., and J.J. performed additional bioinformatic analyses; M.J.L. and A.P. provided clinical correlation data and patient samples; C.C.S. guided all experiments and preparation of the manuscript; and all authors reviewed and approved the final manuscript.

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